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**Assessing relative sensitivity of marine and freshwater bivalves following exposure to copper: Application of classical and novel genotoxicological biomarkers**

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## Abstract

Determination of relative sensitivity of biota following exposures to contaminants including metals is important for environmental protection. Copper (Cu), although biologically essential can be highly toxic to biota if present at higher concentrations in the natural environment. Given its ubiquitous presence within coastal and inland water bodies, we compared Cu-induced genotoxicity in two ecologically important mussel species, the freshwater *Dreissena polymorpha* (DP) and marine *Mytilus galloprovincialis* (MG), along with its tissue specific accumulation. Novel biomarker in terms of induction of gamma H2AX ( $\gamma$ -H2AX) foci, along with comet assay and induction of micronuclei (MN) were used to determine DNA damage response (DDR) in these two species following exposure to a range of Cu concentrations (18, 32, 56  $\mu\text{g L}^{-1}$ ) for 10 days. Concentration-dependent increases in Cu concentration in gill tissue, as determined by Inductively Coupled Plasma Mass Spectrometry (ICP-MS), were paralleled by a greater degree of genotoxicity. An induction of  $\gamma$ -H2AX foci was present in all Cu exposure concentrations, proving this technique to be a sensitive and suitable biomarker of genotoxicity in bivalves. The multi-biomarker approach adopted here suggests firstly that in parallel with MG, which is widely used to assess the health of marine and coastal environment, DP is also suitable representative of inland water bodies, and that there is a similar mechanism of action for the induction of genotoxicity between the two species, following exposure to Cu. Secondly, for genotoxicity assessment a battery of responses could simultaneously be assessed in these two bivalve species. Finally, for adequate protection of the environment it is vital to adopt a multi-biomarker, multi-species approach to determine adverse biological effects to gain a holistic understanding of the real threat posed by contaminants to hydrosphere.

**Keywords:** Metals; Bivalve; DNA damage;  $\gamma$ -H2AX; Bioaccumulation; Genotoxicity

**Abbreviations:**  $\mu\text{g}$ , Microgram;  $\mu\text{M}$ , Micromole;  $\gamma$ -H2AX, Gamma-H2AX; AM, Adductor muscle; Cu, Copper; DAPI, 4',6-diamidino-2-phenylindole, dihydrochloride; DDR, DNA damage response; DG, Digestive gland; DO, Dissolved oxygen; DOC, Dissolved organic carbon; DP, *Dreissena polymorpha*; DSB, Double strand break;  $\text{H}_2\text{O}_2$ , Hydrogen peroxide; ICP-MS, Inductively coupled plasma mass spectrometry; LMA, Low melting point agarose; MG, *Mytilus galloprovincialis*; MN, Micronuclei; NMA, Normal melting point agarose; OST, Other soft tissue; ROS, Reactive oxygen species.

## 1 Introduction

Identification of most sensitive natural species following exposure to anthropogenic contaminants is important from an environmental protection point of view [1-4]. In recent years there has been growing regulatory and scientific concerns for those contaminants which have the potential to exert carcinogenic, mutagenic and endocrine disrupting effects [5]. In this context, it is also emerging that contaminants could simultaneously induce their toxicity in a variety of ways, i.e. the same contaminant could induce carcinogenic, mutagenic, immunotoxic and endocrine disrupting effects [2].

Metals are an important group of ubiquitous contaminants to which biota are exposed in different habitats and ecological niches [4,6]. Exposure to metals can induce a variety of detrimental biological effects via a range of mechanisms, including through the generation of reactive oxygen species (ROS). In addition to damage to cellular components such as lipids and proteins, DNA damage (either directly or through generation of ROS), inhibition of DNA repair capacity and disruption of cell cycle control are of particular concern [5-12]. Although various metals are essential for biological processes, copper (Cu) in particular being important for growth, metabolism and enzymatic activities [13-16], they can be highly toxic to organisms including bivalves at higher concentrations [6,17]. Cu has been the focus of extensive research as a widespread contaminant present in coastal and inland water bodies globally and is considered to be of greater environmental concern compared to other contaminants such as pharmaceuticals [18-19]. Total dissolved Cu in contaminated environments have been found to reach concentrations of 689  $\mu\text{g L}^{-1}$  [20], with permitted levels in England and Wales ranging from 1–28  $\mu\text{g L}^{-1}$  in freshwater (dependant on water hardness), and 5  $\mu\text{g L}^{-1}$  in seawater [21].

The link between metal (in particular Cu) exposure and genotoxicity as determined by induction of micronuclei (MN) and DNA strand breaks (as determined by comet assay), has been well documented in a range of aquatic species, including bivalves [6,17, 22-33]. Induction of DNA strand breaks has been noted at environmentally relevant Cu concentrations in several bivalve species, including *Mytilus* spp. (18-56  $\mu\text{g L}^{-1}$ ), *Perna perna* (37.5-50  $\mu\text{g L}^{-1}$ ), *Limnoperna fortunei* (375-750  $\mu\text{g L}^{-1}$ ) and *Crassostrea gigas* (embryos, 1-20  $\mu\text{g L}^{-1}$ ) [6,17,25,27,30,34].

Species-specific differences in bioaccumulation and resultant biological response following exposure to environmental contaminants have been reported for different marine bivalves [4, 32, 35-36]. Despite the fact that (a) freshwater and marine bivalves are comparable in

their external and internal anatomical structures [37] and (b) contaminants including metals are highly relevant to both marine and freshwater environments, there have been no attempts to compare the relative bioaccumulation and biological responses in these ecologically relevant organisms. The marine, *Mytilus galloprovincialis* (MG) and freshwater mussel, *Dreissena polymorpha* (DP) were chosen for this study as ecologically relevant representatives of both coastal and inland water bodies [37-39]. As sessile, filter feeding organisms, both species are used extensively in monitoring programmes and in toxicological research as accurate bioindicators of their environmental surroundings [40]. The physiology, anatomy and ecology of MG and DP is well understood and their effectiveness within ecotoxicological studies well documented [39]. Bivalves have developed defence and repair mechanisms to compensate for biological damage induced by metal exposures, the key players being antioxidant defence systems, detoxification and sequestration capacity and induction of multixenobiotic resistance proteins (MXR) [41-43]. As noted by Manduzio et al. [42], Al-Subiai et al. [17] and Brooks et al. [32], antioxidant enzymes (e.g. SOD, catalase, GSH) protect cells against biological perturbation (i.e. oxidative damage) by maintaining ROS at non-damaging levels. While the effects of Cu on aquatic organisms are well documented, the relative sensitivity of marine and freshwater bivalves in terms of genotoxic damage, along with defence and repair capacity is yet to be fully explored. As suggested by various authors the use of two (or more) species should be considered as more robust, realistic approach for ecotoxicological studies [3, 4, 44-46].

The extent of damage in individuals evident at higher levels of biological organisation, subsequent to DNA strand breaks (SBs) is dependent on a range of factors including exposure period, contaminant concentrations, rate of uptake, metabolism, accumulation and the efficiency of repair mechanisms [2]. In recent years, immunostaining techniques such as induction of  $\gamma$ -H2AX foci and 53BP1 have been utilised as sensitive markers of DNA double strand breaks (DSBs) [47-48].  $\gamma$ -H2AX plays a key role in DNA damage signalling and repair, along with activation of cell-cycle checkpoints and apoptosis [49]. It is the first step in recruiting and localizing proteins to sites of DSBs formation. Foci are taken as a measure of DNA repair, representing DSBs in a 1:1 manner [50]. The genotoxicity of various contaminants (e.g. metals, nanoparticles, insecticides and radionuclides) using induction of  $\gamma$ -H2AX foci as a biomarker has been displayed in zebrafish (*Danio rerio*) derived ZF4 cells, adult zebrafish liver tissue and retina, and fathead minnow (*Pimephales promelas*) juveniles [51-55]. Concentration-dependent DNA damage in ZF4 cells was evident following aluminium (10-100  $\mu$ M) and cadmium (1- 100  $\mu$ M) exposures [53].  $\gamma$ -

H2AX foci induction followed a similar trend, where number of foci per cell increased with aluminium or cadmium concentration up to 30  $\mu$ M, and then decreased in 50-100  $\mu$ M treatments [53]. Such findings aid to our limited knowledge of DSB-repair mechanisms subsequent to exposure to environmentally relevant contaminants in non-mammalian models. In spite of usefulness of this assay, particularly when validated alongside classical genotoxicity assays (i.e. comet or MN assays), this highly sensitive approach is yet to be applied in aquatic invertebrates.

In light of the above information, following exposure to a range of Cu, the objectives of this study were (a) to investigate the relative tissue specific accumulation of Cu in both bivalve species, (b) to establish a concentration-response curve for genotoxic responses in the adult life stages of the species, (c) to determine relative sensitivity between the two species for genotoxic responses using a range of genotoxicity parameters (i.e. induction of MN, Comet and  $\gamma$ -H2AX foci), (d) to correlate the nominal Cu concentrations in water with bioaccumulation and observed genotoxic responses and (e) to determine potential correlations between different genotoxicity parameters studied. With regards to species variation, we hypothesised firstly that little disparity in genotoxic response will be evident. Secondly, with increased DNA damage (DNA strand breaks and MN formation), a greater induction of  $\gamma$ -H2AX foci will be evident.

## **2 Materials and methods**

### **2.1.1 Chemicals and suppliers**

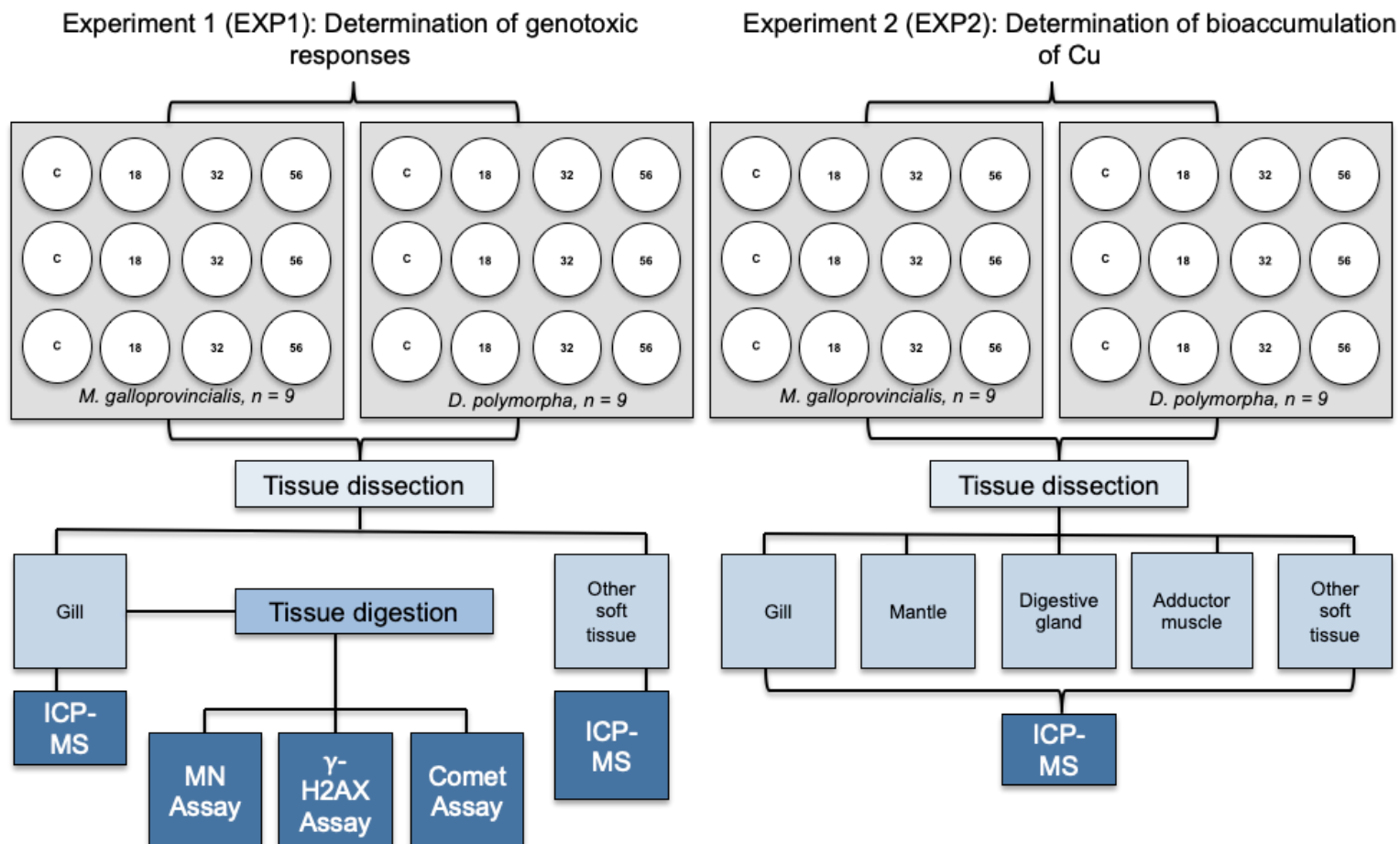
All chemicals and reagents were purchased from Fisher Scientific UK, Anachem Ltd. UK, Sigma-Aldrich Ltd. UK, VWR International Ltd USA or Greiner Bio-One Ltd UK, unless stated otherwise. Product details are mentioned in text as appropriate.

### **2.1.2 Mussel exposure conditions**

Four 10-day Cu exposure experiments were performed between April 2016 and December 2017, the first two to determine genotoxic responses and the latter for tissue specific Cu accumulation measurements. Adult MG (shell length  $44.5 \pm 6.5$  mm) and DP (shell length  $26.7 \pm 4.31$  mm) were collected from Trebarwith strand (as in Dallas et al. [4-5]) and Bude, Cornwall, UK (50.828059, -4.549053), respectively (Vernon et al. [37]). DP was maintained in an artificial river water solution (2M  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 8 mM  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 40 mM, 5 mM  $\text{KNO}_3$ , 0.7 M  $\text{NaHCO}_3$ ), MG in filtered ( $< 10 \mu\text{M}$ ), aerated natural seawater. Mussels were

fed a solution of *Isochrysis galbana* algae (MG; Reed Mariculture, Campbell, CA, USA) or dried *Chlorella* powder, two hours prior to water changes and were kept at a 12:12 photoperiod at 15 °C as described in earlier studies from our laboratory [5,37].

Exposures of bivalves to Cu were staggered by one week for ease of analysis and logistical reasons. Subsequent to a two-week depuration period after collection, individual mussels were transferred into twelve acid washed glass beakers in triplicate (i.e. 1.7 mussels L<sup>-1</sup>), containing 1.8 L<sup>-1</sup> of water and aerated [5, 56]. Individuals were then acclimatised for 48 h prior to exposure. Beakers were labelled and assigned to one of the four treatment groups: 0 (control), 18, 32 and 56 µg L<sup>-1</sup> Cu (as CuSO<sub>4</sub>·5H<sub>2</sub>O, 99% purity), there were three replicates per treatment (Fig. 1). Cu concentrations were selected in accordance to previous work from our laboratory and in line with environmental realistic values [17].



**Figure 1.** Overall experimental design: Experiment 1 (EXP1): Determination of Genotoxic responses; Experiment 2 (EXP2): Determination of bioaccumulation of copper (Cu).

Water changes were performed every alternate day with the appropriate Cu added to meet desired concentration. Due to the invasive nature of DP [57-58], all wastewater was spiked with salt (50 g L<sup>-1</sup>, NaCl) before disposal as to prevent infestation and dispersal. Water samples were taken 1 h after each water change and processed for determination of Cu concentrations as described in section 2.2.2. Water quality parameters were measured daily, and before and after water changes, as outlined on table 1.

**Table 1.** Water quality parameters (pH, temperature, salinity and dissolved oxygen [DO], each parameter measured daily [ $n = 10$ ]) and copper concentrations in water ( $\mu\text{g L}^{-1}$ ,  $n = 6$ ). Data are presented as mean  $\pm$  standard deviation (SD).

Water parameters	EXP 1: Genotoxicity		EXP 2: Bioaccumulation	
	<i>M.galloprovincialis</i>	<i>D.polymorpha</i>	<i>M.galloprovincialis</i>	<i>D.polymorpha</i>
pH	8 $\pm$ 0.11	8 $\pm$ 0.17	8.1 $\pm$ 0.06	8.1 $\pm$ 0.06
Temp (°C)	15.4 $\pm$ 1.22	14.8 $\pm$ 0.28	15.0 $\pm$ 0.24	14.8 $\pm$ 0.32
Salinity	33.2 $\pm$ 0.32	0.3 $\pm$ 0.01	33.1 $\pm$ 0.07	0.3 $\pm$ 0.01
DO (%)	96.5 $\pm$ 2.47	98.8 $\pm$ 0.57	99.0 $\pm$ 0.64	99.0 $\pm$ 0.72
Copper water conc. ( $\mu\text{g L}^{-1}$ )				
Control	0.1 $\pm$ 0	3.1 $\pm$ 0.24	7.3 $\pm$ 0.38	3.6 $\pm$ 0.43
18 ( $\mu\text{g L}^{-1}$ )	12.5 $\pm$ 1.49	14.8 $\pm$ 2.82	20.2 $\pm$ 0.74	19.1 $\pm$ 2.9
32 ( $\mu\text{g L}^{-1}$ )	29 $\pm$ 2.87	25.2 $\pm$ 1.82	30 $\pm$ 1.03	31 $\pm$ 5.41
56 ( $\mu\text{g L}^{-1}$ )	51.6 $\pm$ 6.23	49.8 $\pm$ 3.96	47.5 $\pm$ 2	51.8 $\pm$ 10.06

## 2.2 Determination of Cu concentration in soft tissues and in water samples

### 2.2.1 Copper (Cu) analysis in tissues using Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

Cu determination in tissue samples (i.e. gill, mantle, digestive gland, adductor muscle and 'other' soft tissue) and water was carried out using inductively coupled plasma mass spectrometry (ICP-MS) as described before in publications from our laboratory [5,17,59]. Prior to use, all equipment was acid washed (2% nitric acid, Fisher). Subsequent to dissection, tissue samples were transferred to pre-weighed tubes (15 mL falcon tube, Fisher) and incubated (60 °C, > 48 h), until a constant dry weight was noted. Tubes were re-weighed and recorded for dry weight. To digest, 1 mL concentrated nitric acid (N/2272/PB17, Fisher) was added to each sample. As procedural blanks, 6 tubes were tested alongside containing 1 mL nitric acid, along with a certified reference material



(TORT-2, lobster hepatopancreas). Samples were boiled for 2 h (water bath, 80 °C), or until tissue was fully digested. Once cool, digests were diluted with 4 mL Milli-Q water and stored at room temperature.

### **2.2.2 Determination of Cu in water samples using ICP-MS**

To determine Cu concentrations in water, samples taken on days 1, 5 and 9 (in duplication,  $n = 6$ ) were spiked with 50  $\mu$ L hydrochloric acid immediately after extraction and stored at room temperature until analysis. As described in Dallas et al. [5], indium ( $^{115}\text{In}$ ) and iridium ( $^{193}\text{Ir}$ ) were used as internal standards for both tissue and water samples. Appropriate Cu standards were made to calibrate the instrument before and during analysis of samples [17]. Using appropriate parameters ( $^{63}\text{Cu}$  and  $^{65}\text{Cu}$ ), standards and samples were run using an X Series II ICP-MS (Plasma Quad PQ2 Turbo, Thermo Elemental, Winsford, UK) with PQ Vision 4.1.2 software. Procedural blanks were run every 10 samples.

## **2.3 Sampling procedures**

To determine genotoxicity (EXP1) gill tissue was extracted and stored as followed until use:  $\frac{1}{2}$  gill stored on ice for digestion,  $\frac{1}{2}$  gill stored in pre-weighed tube, placed in 60°C incubator for subsequent ICP-MS determination of Cu content (section 2.2). For Cu accumulation (EXP2) tissue was dissected into gill, mantle, digestive gland, adductor muscle and other soft tissue and processed for ICP-MS as described in section 2.2.

## **2.4 Biological assays**

### **2.4.1 Isolation of gill cells for genotoxicity assays**

The procedure to obtain gill cells for genotoxicity assays was adopted from previous studies [60]. Briefly, 1 mL dispase II solution (1.6 mg dispase powder per 1 ml of HBSS, Sigma) was added per glass vial (20 mL, Fisherbrand™ Borosilicate Glass). This was transferred into a pre heated (35 °C) water bath for 5 min prior to use. Tissue was transferred to vials for 30 min (37 °C); shaken every 10 min. Following incubation, cell suspension was centrifuged (1200 rpm, 5 min). Supernatant was then used in subsequent assays, providing cell viability, checked using the Trypan Blue exclusion dye assay [61], was <90% across all treatments (data not included).

### **2.4.2 Comet assay to determine DNA strand breaks**

Determination of DNA strand breaks using the single cell gel electrophoresis or the comet assay was conducted as described elsewhere in detail [62]. Prior to *in vivo* exposure of mussels to Cu, the comet assay was validated using isolated gill cells using a range of concentrations of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>, 100 µL, 0, 5 or 500 µM in PBS, 1 h) as a reference agent, as described elsewhere [5]. Briefly, cell suspension of gill cells (as described in 2.4.1, 150 µL) was used immediately for the comet assay. A cell-agarose suspension (1% LMA, Sigma) was pipetted in duplicate, onto a pre-coated (1% NMA in TAE) slide. After setting in the fridge (1 h), slides were placed in a chilled lysis buffer (1 h, 4 °C). Following lysis, cells were given a 20 min DNA denaturation in electrophoresis buffer (0.3 M NaOH and 1 mM EDTA, at pH 13) and then electrophoresis for 25 min at 21 V and 620 mA. Slides were placed in a neutralisation buffer (0.4 M Tris Base, Sigma) for 5 min and then chilled distilled water for another 5. Cells were stained with ethidium bromide (20 µL of 20,000 µg L<sup>-1</sup>) and scored using an epifluorescent microscope (DMR; Leica Microsystems, Milton Keynes, UK). 100 cells per slide (50 cells per microgel) were quantified using the Comet IV imaging software (Perceptive Imaging, Bury St Edmunds, UK) software. The software provides results for different parameters, % Tail DNA was considered the most reliable to present the results [63].

#### **2.4.3 Analysis of micronuclei (MN) induction**

The procedure to prepare slides for the analysis of micronuclei (MN) was adopted as described elsewhere in detail [28, 5], with minor modifications. Cell suspension (50 µL) was smeared onto a coded slide (1 h, 4 °C) and were chilled to allow cells to adhere. For fixation, ice-cold carnoys solution (1 mL, 75% methanol, 25% glacial acetic acid) was gently pipetted onto slide and left for 20 min. Fixative was carefully tipped off and slides allowed to dry overnight (room temp). To score, slides were stained with 20 µL ethidium bromide (20 µL of 20,000 µg L<sup>-1</sup>). 500 cells were scored per slide using a fluorescent microscope (as in section 2.4.2), slides were scored at random to prevent bias. Micronuclei classification was in accordance to Venier et al. [74] and Bolognesi and Fenech [28]. Results are reported as mean MN per 1000 cells, in keeping with other data from our research group [5].

#### **2.4.4 Induction of γ-H2AX foci**

Phosphorylation of the histone protein H2AX is indicative of DNA DSBs [65]. Being highly conserved between species [66] we were able to utilise antibodies from differing species to measure γ-H2AX foci induction in marine and freshwater bivalves [67]. Prior to Cu

exposure,  $\gamma$ -H2AX response was optimised and validated under *in vitro* conditions in numerous cells, including haemocytes, gill and digestive gland cells of both the bivalve species using standardized techniques on human cells in our laboratory conditions [48,68]. Optimisation of the gamma-H2AX assay in gill cells was performed using a range of concentrations of hydrogen peroxide ( $\text{H}_2\text{O}_2$ , 100  $\mu\text{L}$ , 0, 5, 50 or 500  $\mu\text{M}$  in PBS, 1 h) as a reference agent [5,69]. Briefly, gill tissue was digested as in section 2.4.1, and pooled to reduce inter-individual variability. Aliquots (150  $\mu\text{L}$ ) of cells were transferred into microcentrifuge tubes and spun (775 g, 2 min, 4  $^\circ\text{C}$ ). Once supernatant was removed and discarded (leaving approx. 10  $\mu\text{L}$ ),  $\text{H}_2\text{O}_2$  was added to the cellular pellet. Following incubation (1 h, 4 $^\circ\text{C}$ , dark) samples were spun (as before), supernatant removed and samples processed as detailed below.

Briefly, cells (100  $\mu\text{L}$ , section 2.4.1) were secured onto coverslips using a slide centrifuge (Cytospin 4, Thermo Fisher Scientific Inc., Waltham, MA, USA, 800 rpm) for 5 min. Coverslips were placed in individual wells (6 well plate, sterile, Greiner Bio-One), chilled (20 min, 4  $^\circ\text{C}$ ) to allow adhering and fixed with ice-cold Carnoys solution for 20 min at room temperature (75% methanol, 25% glacial acetic acid, 1 mL per well). Subsequent to fixation, coverslips were rinsed with PBS (Dulbecco, Fisher) in triplicate.

Cells were permeabilised (10 min, 0.5% Triton X-100 in PBS, room temp), blocked (1.5 h, normal goat serum, 60  $\mu\text{L}$  per coverslip, G9023, Sigma) and rinsed in triplicate with 0.1% Triton X-100/PBS. Cells were incubated overnight (4  $^\circ\text{C}$ ) with the primary antibody (60  $\mu\text{L}$  per slide, 1:10000 in 0.1% Triton X-100/PBS, anti-GamaH2H [ $\gamma$ -H2AX], Merck Millipore, UK). Procedural blanks were run alongside samples, with no primary antibody. Following a rinse (in triplicate, 0.1% Triton X-100/PBS) cells were incubated in the dark with a secondary antibody, at room temperature (1 h, 60  $\mu\text{L}$  per slides, 1:1000 in 0.1% Triton X-100/PBS, Anti-IgG secondary antibody), and then rinsed as before.

Cells were counterstained with DAPI (1  $\mu\text{g}$  10  $\text{mL}^{-1}$  PBS) for 10 min in the dark. Subsequent to rinsing (in duplicate, distilled water), coverslips were gently removed from well plates, tilted to remove excess liquid and mounted onto labelled slides. Slides were scored (50 cells per individual) by counting number of foci per cell using a fluorescence microscope (Nikon fluorescence microscope, 60x magnification). As described by Festarini et al [70], cell nuclei were located with an appropriate DAPI filter, and a FITC filter set for the FITC signal of the  $\gamma$ -H2AX primary antibody. All slides, including procedural blanks were coded and scored at random.

## 2.5 Statistical analysis

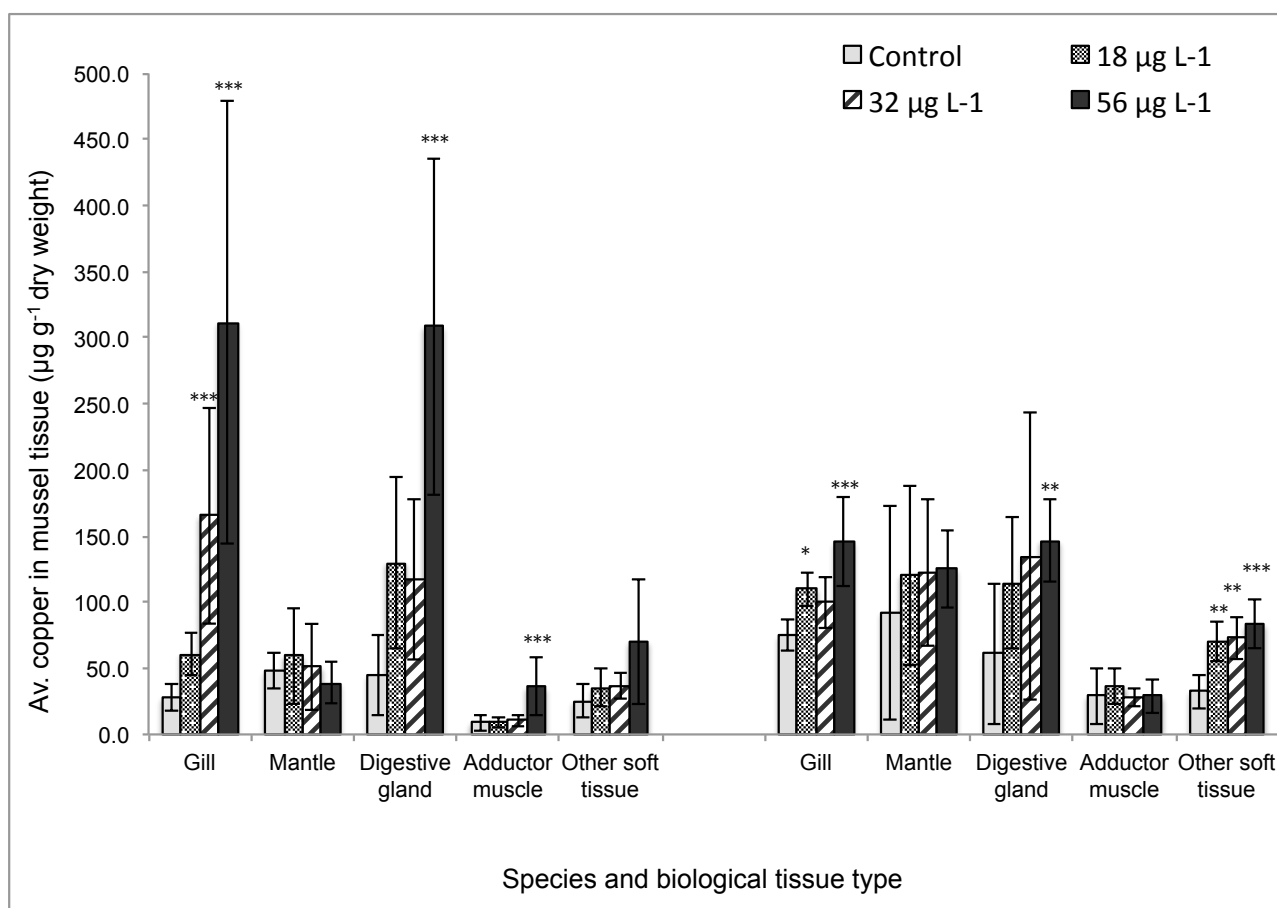
All statistical analyses were carried out using the statistical software R (RStudio, R 3.4.3 GUI 1.70 El Capitan build (7463), <https://www.r-project.org/>). All data was checked for normality distribution (Shapiro-Wilk test) and homogeneity of variances (Levene's test), with visual examination of QQ-plots. The non-parametric Kruskal-Wallis test was used if assumptions were not met; comparison between treatment groups was determined using a Dunn's pairwise comparison with Bonferroni correction. Where assumptions were met, a one-way ANOVA was run with Tukey's post hoc tests. Comparison between treatment groups was determined using a Wilcoxon rank sum test with Holm-Bonferroni correction. Regression analysis, using a Pearson correlation coefficient was used to determine any correlations between variables. Level of significance for all tests was set at  $p < 0.05$  (\*) and data presented as mean  $\pm$  standard deviation, unless otherwise stated.

## 3 Results

No spawning of mussels occurred during the duration of the experiments and mortality remained low throughout, with one fatality in the highest Cu treatment (MG,  $56 \mu\text{g L}^{-1}$ , EXP1). Metal concentration and water quality measurements are displayed in table 1, results of the ICP-MS analysis confirmed that achieved values were in line with expected Cu concentrations across all treatments.

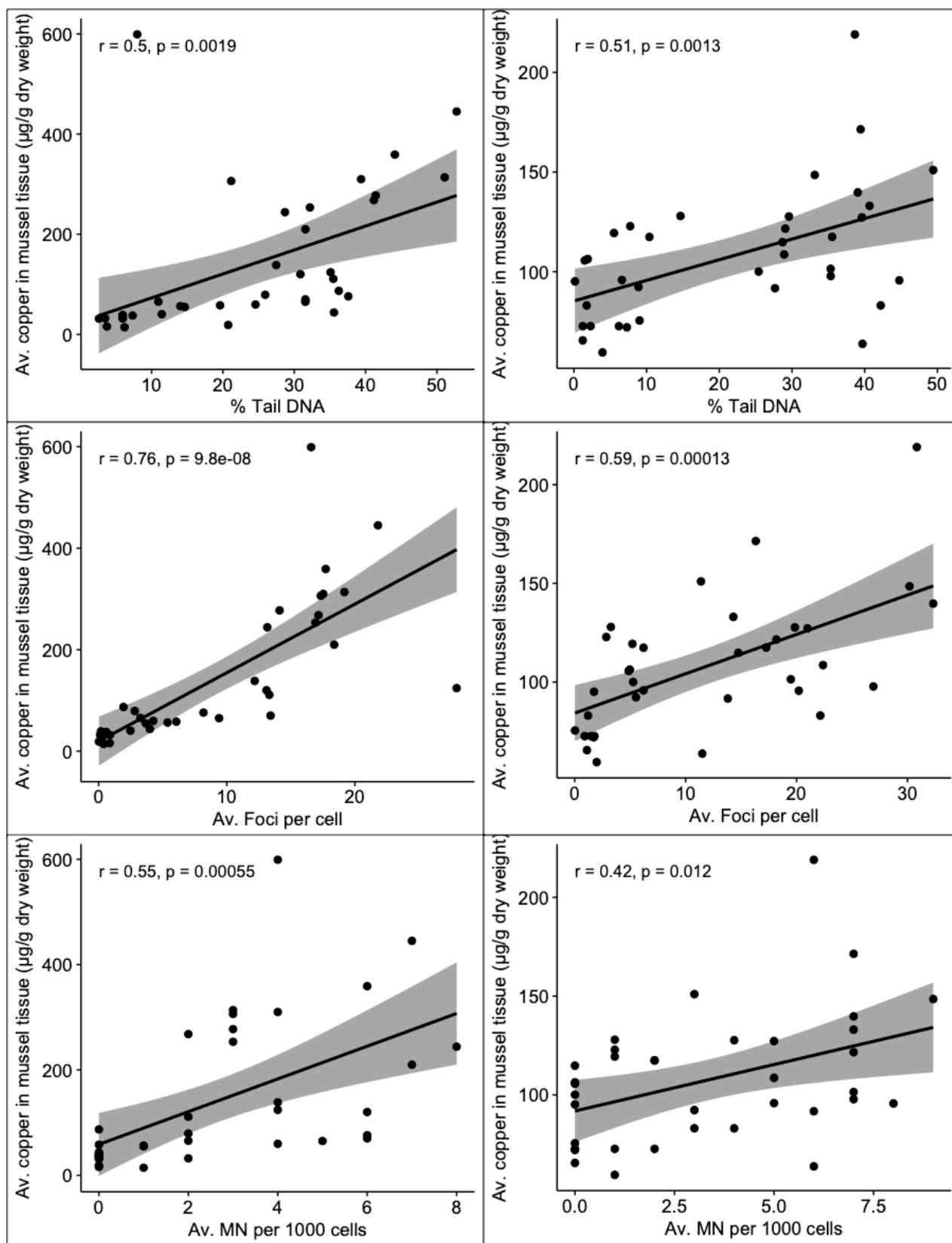
### 3.1 Tissue specific Cu accumulation

After a 10-day exposure a substantial accumulation of Cu in both bivalve species was observed. Fig. 2 highlights the variable nature of tissue specific uptake. Cu accumulation occurred in a concentration dependant manner in all tissues but the mantle of both species, MG 'other' soft tissue, and DP adductor muscle. Cu uptake varied between tissue, and between species. Concentrations ranged between  $8.7$  and  $311.4 \mu\text{g g}^{-1}$ , with the highest levels evident in gill and digestive gland, independent of species. In the highest treatment group ( $56 \mu\text{g L}^{-1}$ ), accumulation varied in the order of gill > digestive gland > other soft tissue > mantle > adductor muscle in MG, and digestive gland > gill > mantle > other soft tissue > adductor muscle in DP. In terms of whole soft tissue (sum of all tissue), DP had a greater degree of accumulation than MG in all but the highest treatment group. In the  $32 \mu\text{g L}^{-1}$  treatment, the accumulation of Cu in DP ( $458 \mu\text{g g}^{-1}$ ) was 1.2 times higher than in MG ( $382 \mu\text{g g}^{-1}$ ), and approx. 2 times higher in control treatments.



**Figure 2.** Tissue specific accumulation of copper in *M. galloprovincialis* (left) and *D. polymorpha* (right), microgram per gram of mussel tissue (dry weight) in control and exposed treatment groups. Asterisks (\*, \*\* or \*\*\*) are indicative of significant differences ( $p < 0.05$ , 0.01, 0.001) from the corresponding control. SD is standard deviation of mean data.  $n=9$ .

Biological response clearly correlated with the level of Cu accumulation in gill tissue, where the highest genotoxic response was found in mussels exposed to the two highest Cu concentrations (Fig. 3).



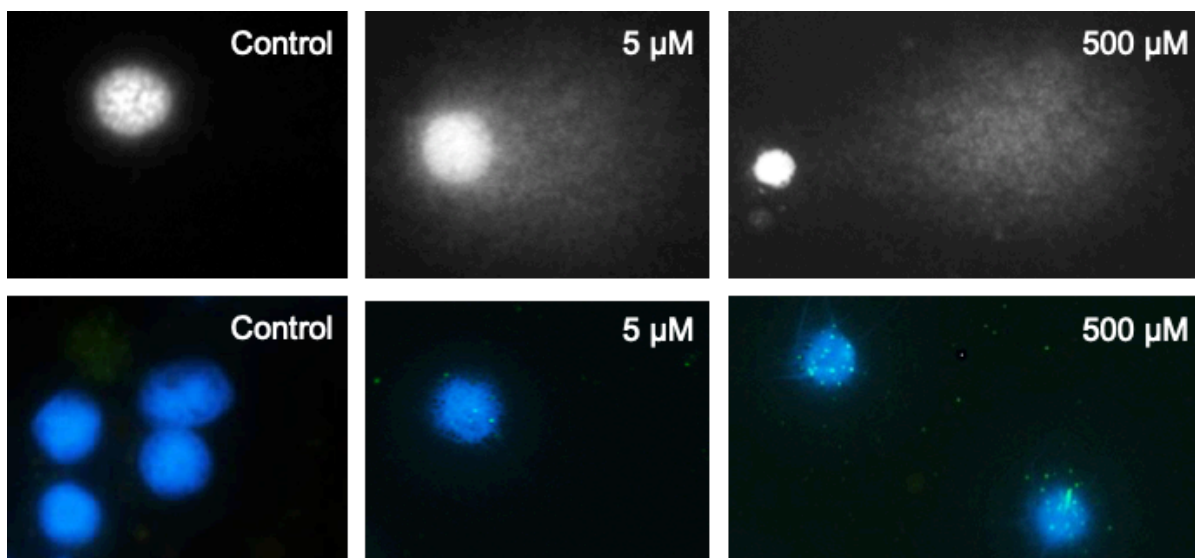
**Figure 3.** Pearson's correlation analyses of Cu accumulation in gill tissue. Top to bottom: DNA damage (% tail DNA), induction of  $\gamma$ -H2AX foci and micronuclei (MN) formation in *M. galloprovincialis* (left) and *D. polymorpha* (right).  $n = 9$ .

### 3.2 Genotoxic effects and repair capacity in mussel gill cells

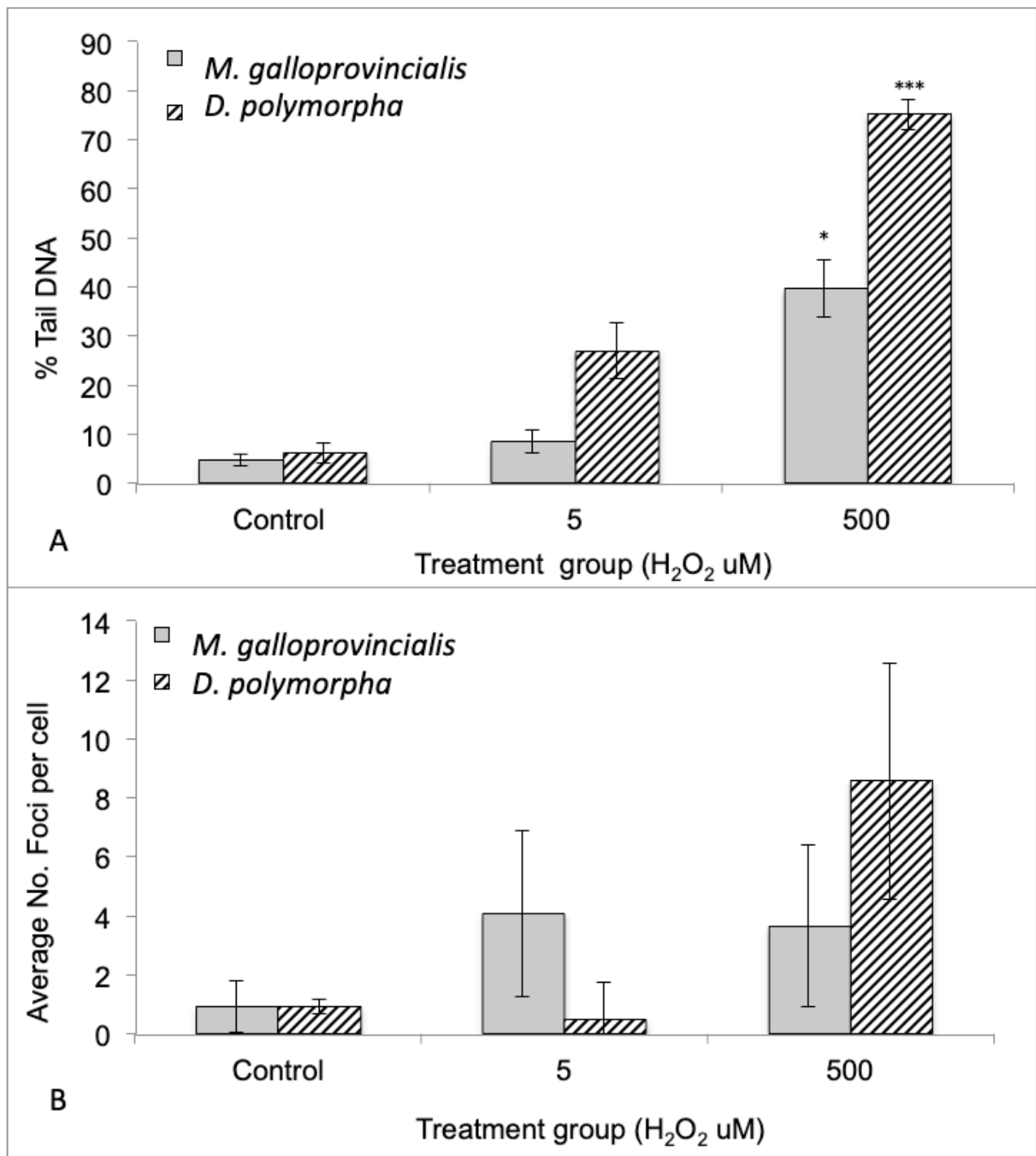
#### 3.2.1 *In-vitro* validation of comet and $\gamma$ -H2AX assays

Fig. 4 provides the representative photographs of the DNA damage (as determined by the comet assay) and induction of  $\gamma$ -H2AX foci in gill cells of DP following *in vitro* validation studies using hydrogen peroxide ( $\text{H}_2\text{O}_2$ ). Fig. 5 shows the mean (+ S.D) number of  $\gamma$ -H2AX foci, and % Tail DNA in MG and DP gill cells following exposure to varying concentrations of  $\text{H}_2\text{O}_2$ . In both species,  $\text{H}_2\text{O}_2$  produced a concentration-dependent increase for increased % Tail DNA ( $p = 0.01$  and  $p = 0.001$ ) and for the number of foci per cell ( $p = 0.01$  and  $p = 0.04$ ) compared to controls for both the species (Figure 5A and B).

<Insert Figure 4 and 5>



**Figure 4.** Photomicrographs showing DNA damage, as measured by % Tail DNA (top) and induction of  $\gamma$ -H2AX-foci (bottom) in gills cells of *D. polymorpha* following a 1 h exposure to varying  $\text{H}_2\text{O}_2$  concentrations.

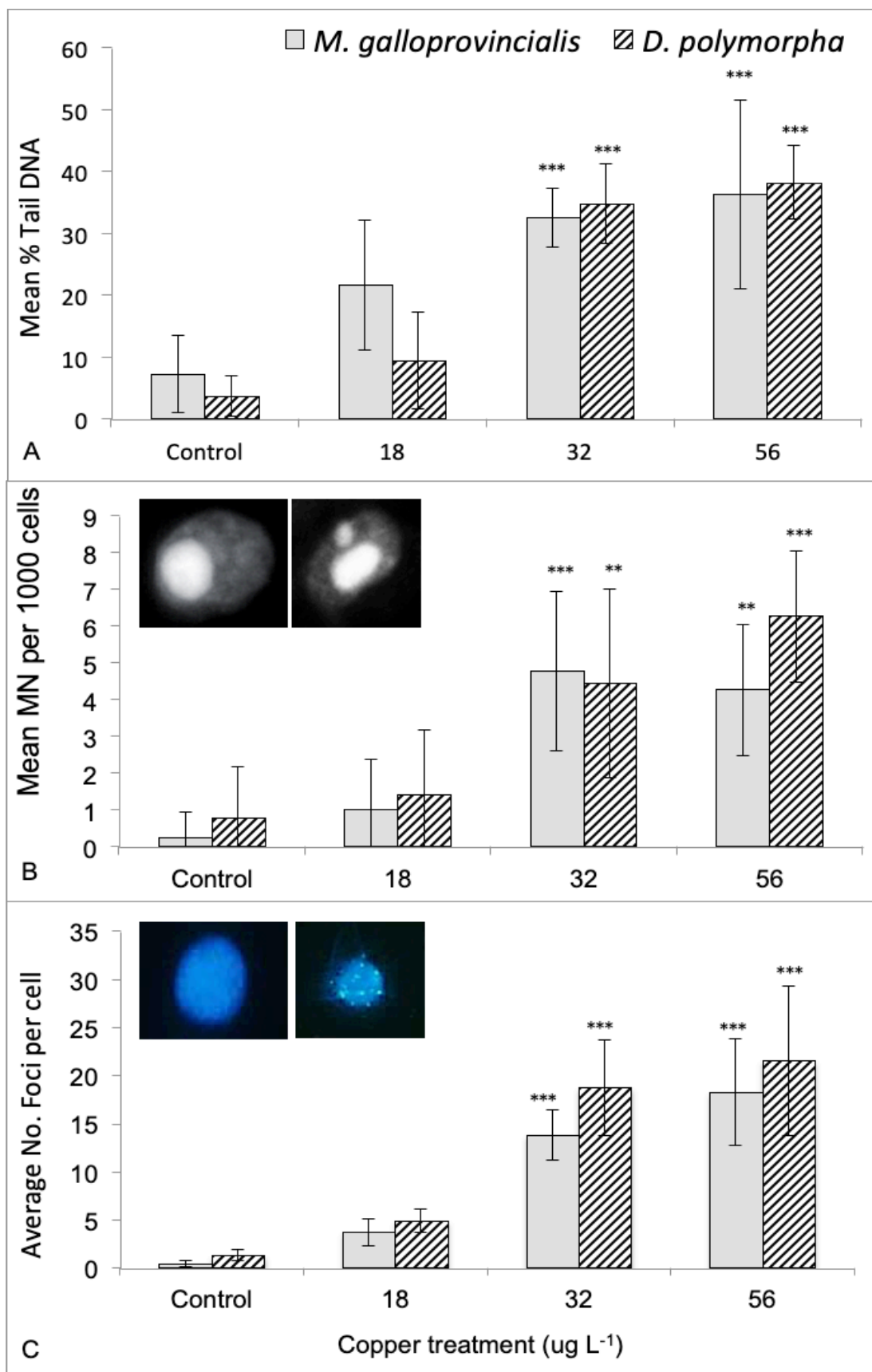


**Figure 5.** Graphs to show (A) DNA damage (Tail % DNA) and (B) average number of  $\gamma$ -H2AX foci in *M. galloprovincialis* and *D. polymorpha* gill cells following exposure to varying concentrations of hydrogen peroxide. SD is standard deviation of mean data. Asterisks (\*, \*\* or \*\*\*) are indicative of significant differences ( $p < 0.05$ ,  $0.01$ ,  $0.001$ ) from the corresponding control.  $n=4$ .



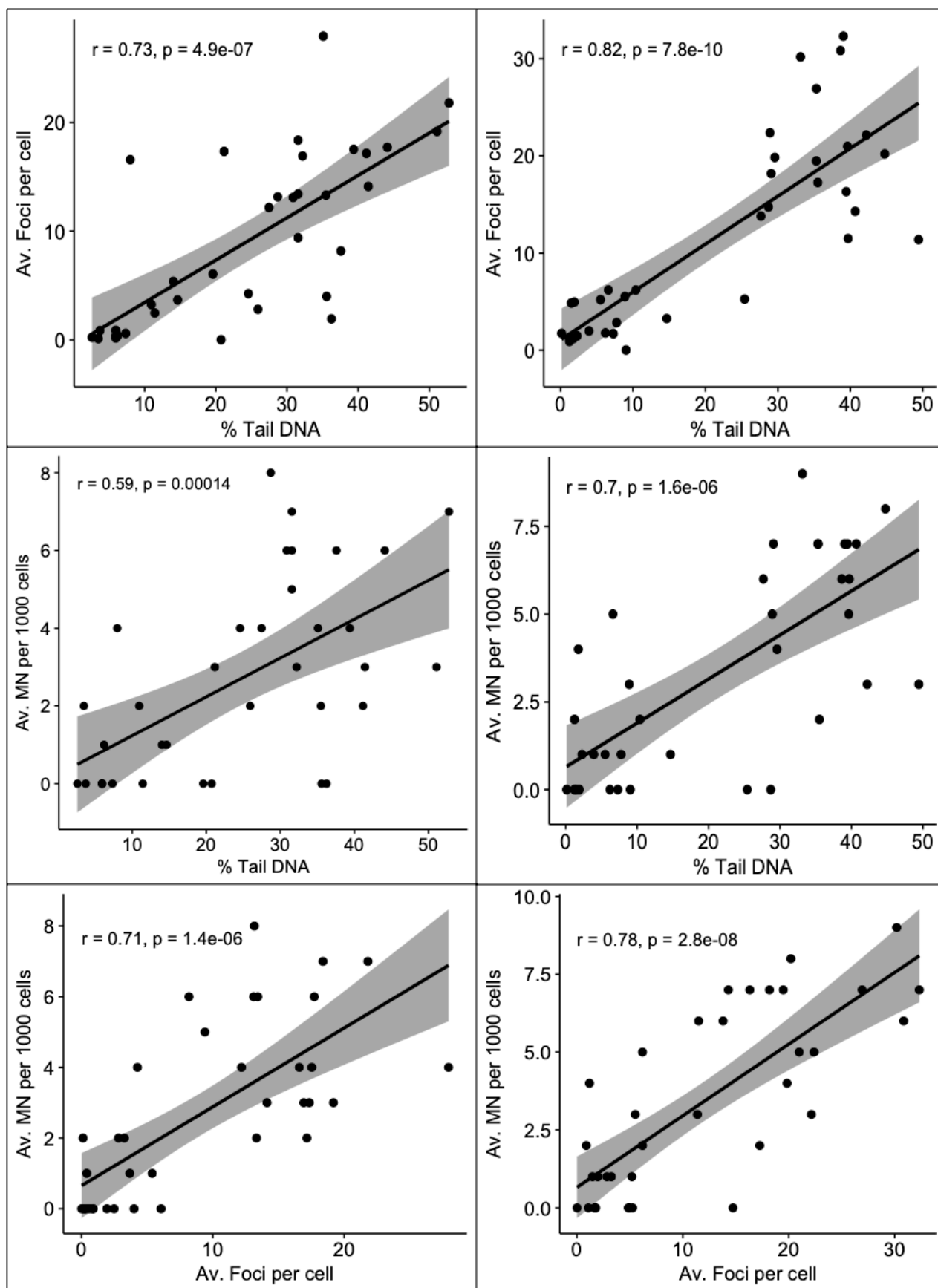
### 3.2.2 Genotoxic response following in vivo exposures to Cu

Fig. 6A, B and C show the mean (+ S.D) % tail DNA damage, micronuclei per 1000 cells and  $\gamma$ -H2AX foci induction respectively in the gill cells of the two species following exposure to varying Cu concentrations for 10 days. Both the species showed genotoxic effects following Cu exposure compared to controls. Independent of species a concentration dependant increase was evident across all genotoxic biomarkers in response to Cu ( $p < 0.001$ ).



**Figure 6.** Genotoxic responses in *M. galloprovincialis* and *D. polymorpha* gill cells following a 10 day exposure to copper (Cu). (A) DNA damage (% tail DNA), (B) Induction of micronuclei (MN) and (C) induction of  $\gamma$ -H2AX foci Asterisks (\*, \*\* or \*\*\*) are indicative of significant differences ( $p < 0.05$ , 0.01, 0.001) from the corresponding control. SD is standard deviation of mean data. Images show (left) control cell and (right) damaged cell.  $n=9$ .

Although a genotoxic response was evident in the lowest Cu treatment for all biomarkers studied, a significant response was evident only for 32 and 56  $\mu\text{g L}^{-1}$  treatments compared to the controls. Interestingly, there was no significant difference in response between the 32 and 56  $\mu\text{g L}^{-1}$  treatments in either species. In the highest concentration (i.e. 56  $\mu\text{g L}^{-1}$  Cu) the average level of induced  $\gamma$ -H2AX foci per cell was  $18 \pm 6$  and  $22 \pm 8$  foci per cell in MG and DP, compared with  $0.4 \pm 0.3$  and  $1 \pm 0.6$  foci per cell for control treatments. For both species, the observed response for  $\gamma$ -H2AX showed a strong correlation with DNA damage ( $p < 0.001$ ) and MN formation ( $p < 0.001$ , Fig. 7). The % tail DNA in the highest treatment averaged around 37% (both species), as expected a low degree of damage was evident in control treatments. DNA damage in individuals exposed to the highest Cu concentrations was 5 and 9.5 times higher in MG and DP, in comparison to the control. In terms of species comparison, despite disparity in Cu accumulation among the tissues, there was little variation in genotoxic response in the cells. The only significant variation occurred between  $\gamma$ -H2AX foci induction in control cells ( $p < 0.05$ ).



**Figure 7.** Pearson's correlation analyses. Top to bottom: % tail DNA and induction of  $\gamma$ -H2AX foci; % tail DNA and induction of MN and induction of MN and  $\gamma$ -H2AX foci in *M. galloprovincialis* (left) and *D. polymorpha* (right).  $n=9$ .

## 4 Discussions

### 4.1 Tissue-specific Cu accumulation

It is well accepted that following uptake, waterborne contaminants are not uniformly distributed among the tissues due to their inherent metabolic capabilities [4,17, 37, 71-74]. The presence of metals in the sediments has also been correlated with their accumulation in soft tissues of bivalves under field conditions [4]. As expected, in our study Cu concentrations varied among the tissues [75-78]. The highest Cu concentrations were found in the gill and digestive gland, particularly in the MG 56  $\mu\text{g L}^{-1}$  treatment. In this treatment, 40% (MG) and 28% (DP) of accumulated Cu was located in the digestive gland, this is in contrast to previous report where Cu in DP had been shown to predominately accumulate in the foot (included in other soft tissue in this study), followed by gill and digestive organs [79]. Mussel digestive systems are well known to harbour contaminants via dietary uptake pathways [22,74, 80-81]. Apart from inherent metabolic differences, uptake and bioaccumulation is dependent on many factors including bioavailability, uptake mechanism and biological factors (e.g. weight, gender, reproductive stage, feeding habits etc.). These factors may help explain disparity between the studies [2].

In line with findings from Sanders et al. [82], Zorita et al. [83] and Al-Subiai et al. [17], a high degree of Cu bioaccumulation was evident in gill tissue of both species. Bivalve gills, due to proximity to surrounding media and therefore the primary sites of uptake of dissolved Cu, are often regarded as a key tissue of interest in ecotoxicological studies. In contrast to Al-Subiai et al. [17], who found reduced Cu accumulation in 56  $\text{mg L}^{-1}$  compared to 32  $\text{mg L}^{-1}$  treatment across all tissue (adductor muscle, digestive gland, gills), in our study accumulation increased in a concentration dependant manner in accordance to external Cu concentration (all but the MG mantle and DP adductor muscle tissue, Fig. 2). These differences may result from varying experimental procedures, including shorter exposure length or varying feeding regimes (i.e. individuals fed/not fed). Cu bioaccumulation and subsequent biological response in *Mytilus* sp. has been investigated by Brooks et al. [32], who found an increased rate of bioaccumulation in *M. trossulus* compared to *M. edulis/galloprovincialis* (4 d, 500  $\mu\text{g L}^{-1}$ ), and a higher prevalence of MN in *M. edulis* compared to *M. trossulus*. The mussels included in this study were collected from three different geographical locations (i.e. Norway and the Basque country, Spain). Hybridization and introgression of geographically dispersed species could play an important role in the bioaccumulative potential of contaminants, and may explain the differences found between biological studies [17,32, 84]. A more complete introgression

analysis that is not assessed by the Glú-5 gene (nuclear DNA marker used to characterise *Mytilus* sp.) may help to identify species differences that affect contaminant uptake [85-86].

Whole soft tissue Cu concentrations were reflective of exposure with accumulation occurring in a concentration dependant manner. Interestingly, in all but the highest treatment DP showed a greater degree of Cu accumulation in whole soft tissue, uptake in DP also appeared to be more evenly distributed across specific tissues. It is important to note that differential Cu speciation in water bodies may affect its bioavailability and subsequent toxicity to aquatic biota. The physical and chemical form of Cu, with focus on the toxic ionic form ( $\text{Cu}^{2+}$ ) varies between salt and freshwater environments, becoming more abundant at lower salinities [87]. The influence of water parameters (i.e. pH, salinity, dissolved organic carbon [DOC], alkalinity) in affecting Cu bioavailability and toxicity may explain disparities present within our data, along with differential physiology [88-90]. As certain parameters (e.g. DOC) were not determined during this experiment, we are unable to determine if the species disparity resulted from varying water chemistry, especially DOC or differential species sensitivity. In both species independently, however, correlation between accumulations of Cu in soft tissues with increasing genotoxicity in gill cells were evident.

#### **4.2 Cu induced genotoxicity in gill cells**

The capacity of Cu to induce chromosomal damage in a range of cell types has previously been reported in MG [32, 91], and in DP in response to a range of contaminants [92-94]. As mentioned earlier, Cu accumulation in mussel tissue is significantly correlated with the adverse genotoxicological effects noted in both the species (Fig. 3). Cu toxicity in marine bivalves has been demonstrated extensively in scientific literature [6,17, 24, 95], along with freshwater species [96-98]. In line with previous studies, significant effects (i.e. DNA strand breaks and MN induction) were evident in both marine and freshwater mussels exposed to the highest Cu concentrations (32 and 56  $\mu\text{g L}^{-1}$ ), with a 4-9 fold increase in DNA damage relative to controls. The genotoxicity of Cu in mussel gill cells may be related to the overproduction of ROS, leading to oxidative damage in the form of single and double strand DNA breaks (SSBs and DSBs), base modifications or oxidation of bases [8]. Furthermore,  $\text{Cu}^{2+}$  is known to bind to DNA, forming adducts [99].

While MG individuals showed greater Cu concentrations in gill tissue there was a high degree of comparability between species response, suggesting that Cu toxicity is not necessarily related to accumulation. In terms of the comet assay results, both species

showed around 34-38% damage (% Tail DNA) in two highest Cu concentrations. Background levels of DNA damage in the controls were around  $7 \pm 6$  (MG) and  $4 \pm 3$  % (DP, % Tail DNA), relatable to previous findings [3, 5, 71,100], this suggests general good health of the unexposed (control) individuals. Interestingly, Cu genotoxicity was not significantly evident at the lowest concentrations ( $18 \mu\text{g L}^{-1}$ ), in either species. This is contrast to Anjos et al. [101] who found significantly increased DNA damage in sea anemone (*B. cangicum*) pedal disk cells exposed to much lower Cu concentrations ( $7.8 \mu\text{g L}^{-1}$ , 24 h). Our data was also in contrast to that of Al-Subiai et al. [17], who noted significantly increased DNA damage in *Mytilus edulis* at  $18 \mu\text{g L}^{-1}$ , following a 5 day Cu exposure. As mentioned above, several biological and physico-chemical factors could account for these differences [2].

As previously mentioned, direct species comparison is limited due to differing water chemistry, potentially altering bioavailability. In addition, tissue Cu concentration is not necessarily a reliable indicator of toxicity. It is only a proportion of metal that interacts with sensitive target molecules (i.e. DNA) that induces a toxic effect. Despite this, it is clear that even at low tissue concentrations a genotoxic response is present in both species. Larsson et al. [84] found a lack of difference in response to environmental stressors between marine mussels collected from reference and contaminated sites (i.e. sewage treatment plants, harbours) around the Baltic Sea region. The authors suggest that the presence of strong introgression between the two *Mytilus* taxa, along with adaptation to the specific environmental conditions could have accounted for this lack of differential sensitivity [84]. In our study, the similarity in DNA damaging effects suggests a similar mechanism of action in response to pollutants in the two species.

This study highlights the potential of zebra mussels (DP) as a freshwater equivalent to *Mytilus* species. Due to their ubiquitous, invasive nature they are regarded as a fairly tolerant, insensitive species [96]. As with *Mytilus* spp., a range of cell types from gill to haemocytes can be successfully utilised in biological assays. As expected, in this study damage to DNA and MN formation was significantly correlated, in both species. Previous studies from our laboratory have reported significant correlations between induction of MN and DNA strand breaks in mussels and sea stars following exposures to environmentally relevant metals and pharmaceuticals [3,5]. In the present study, it was interesting to note very good correlations between induction of  $\gamma$ -H2AX foci with DNA strand breaks and micronuclei (Fig. 7). Such a relationship is increasingly recognized in mammalian in vitro studies [102]. To our knowledge, such correlations between different genotoxicity parameters, especially in aquatic organisms have not been reported previously. The

combined use of these biomarkers allows for holistic determination of the genotoxic damage induced by environmental agents which could be applied to other natural species.

One interesting aspect observed in this study is while no significant difference in terms of DNA damage is evident between the highest concentrations (32, 56  $\mu\text{g L}^{-1}$ , both species), there is a slight increase in  $\gamma$ -H2AX foci in the 56  $\mu\text{g L}^{-1}$  treatment. This could be a result of increased DSBs in the highest treatment, as opposed to less detrimental DNA lesions (i.e. single strand breaks) at lower Cu concentrations.  $\gamma$ -H2AX foci were present in the lowest Cu concentrations (18  $\mu\text{g L}^{-1}$ ), where the genotoxicity of Cu was not significantly apparent. Our data suggests that while DNA damage was not evident using the comet or MN assays, possibly due to lack of sensitivity,  $\gamma$ -H2AX is being recruited to damaged sites, in turn recruiting other DNA repair machinery even at low Cu concentrations.

In terms of relative sensitivity of the comet and induction of  $\gamma$ -H2AX assays, one major drawback of the comet assay is its inability to discriminate between DNA lesions, such as single and double SBs, alkali labile sites and DNA interstrand crosslinks [63,103-106]. DSBs are considered to be most detrimental form of damage, they may be repaired or result in apoptosis and/or mutation. Induction of  $\gamma$ -H2AX is increasingly used as a biomarker in combination with classical and molecular techniques in mammalian systems [48,68,102]. Whilst these techniques are considered to be simple and rapid (fast analysis time) in comparison to many other established methodologies [106], their relative sensitivity and effectiveness have not been compared sufficiently in ecotoxicological studies. In this study, we compared the classical and novel techniques, examining their sensitivity as well as cost/time effectiveness. Although the biological damage measured by Comet and induction of  $\gamma$ -H2AX assays are mechanistically different (one reflecting SSBs/DSBs, alkali labile site and another only DSBs), the alkaline comet assay appears to be more suitable for larger sample sizes that have high levels of DNA damage. On the other hand induction of  $\gamma$ -H2AX foci, due to its high sensitivity could be considered more suitable for studies which aim to determine DNA damage at lower levels of exposure, to those genotoxins capable of effectively inducing DSBs. This is particularly so as at higher levels of damage, induced foci can overlap making the scoring difficult and time-consuming. Overall, induction of  $\gamma$ -H2AX foci has proved useful as a highly sensitive technique for detecting low-levels of DNA damage, its usefulness in ecotoxicological research, when combined with more classical techniques is clear.



Cu, as a model toxic metal is known to induce various types of damage to DNA and chromatin with potential pathophysiological consequences [8,10]. As mentioned earlier, one of the important mechanisms of induction of damage by Cu is via generation of reactive oxygen species (ROS or free radicals) inducing oxidative stress to biomolecules including DNA [8,10]. Modified comet assays using bacterial enzymes (e.g. FPG, Endo III) have been used by different workers to determine DNA oxidation in fish and mussels [5,107]. It would have been useful to determine DNA oxidation using the modified comet assay in this study as well, to determine relative contribution of DNA oxidation. This was however not feasible due to logistical problems. In addition, Cu in common with other toxic metals (e.g. As, Co, Cd, Ni) could also interfere with DNA repair processes and cell cycle control [11]. In common with mammalian studies, elucidation of these fundamental processes in aquatic organisms following exposures to environmental contaminants also warrants attention. Interpreting these highly conserved processes would help to further strengthen human and environmental links.

## 5 Conclusions

Our study has been the first to compare tissue specific accumulation and genotoxic effects following exposure to Cu in marine and freshwater bivalve gill cells. The zebra mussel, DP, is increasingly being utilised as a freshwater counterpart of *Mytilus* spp. in biomonitoring and ecotoxicological research. Our data highlights a clear relationship between external (water) and internal Cu concentrations. The capacity to concentrate contaminants within tissue makes MG and DP suitable bioindicator species to assess environmental health. Cu induced comparable chromosomal and DNA damage in both mussel species, despite variable bioaccumulation of Cu into gill tissue. Furthermore, induction of  $\gamma$ -H2AX foci formation was successfully applied as a useful biomarker of contaminant induced genotoxicity. The usefulness of this assay, particularly when applied alongside more classical, established techniques such as MN and comet assays is evident.

The relative sensitivity of species could vary depending upon the mechanisms or mode of actions of genotoxicants. For use in biomonitoring, it is important to validate the  $\gamma$ -H2AX and other assays further in a wider range of aquatic biota, to well established genotoxic pollutants with different mechanisms or mode of action, before their applications in the field conditions. While we cannot definitively associate the relative comparability in genotoxic response to differential species sensitivity following exposure to a novel metallic toxicants, our results suggest that even low, environmentally realistic Cu concentrations have the

potential to cause stress to some bivalve molluscs. For adequate protection of coastal and inland water bodies, future research would benefit from using a multi-species, multi-biomarker approach when investigating adverse effects at varying levels of biological organisation to gain a true understanding of the real environmental threat of the contamination to aquatic biota.

**Conflict of interest:**

Authors declare no conflict of interest.

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## **Vernon and Jha : Figure legends and Table Captions**

**Figure 1.** Overall experimental design: Experiment 1 (EXP1): Determination of Genotoxic responses; Experiment 2 (EXP2): Determination of bioaccumulation of copper (Cu).

**Figure 2.** Tissue specific accumulation of copper in *M. galloprovincialis* (left) and *D. polymorpha* (right), microgram per gram of mussel tissue (dry weight) in control and exposed treatment groups. Asterisks (\*, \*\* or \*\*\*) are indicative of significant differences ( $p < 0.05, 0.01, 0.001$ ) from the corresponding control. SD is standard deviation of mean data.  $n=9$ .

**Figure 3.** Pearson's correlation analyses of Cu accumulation in gill tissue. Top to bottom: DNA damage (% tail DNA), induction of  $\gamma$ -H2AX foci and micronuclei (MN) formation in *M. galloprovincialis* (left) and *D. polymorpha* (right).  $n=9$ .

**Figure 4.** Photomicrographs showing DNA damage, as measured by % Tail DNA (top) and induction of  $\gamma$ -H2AX-foci (bottom) in gills cells of *D. polymorpha* following a 1 h exposure to varying  $H_2O_2$  concentrations.

**Figure 5.** Graphs to show (A) DNA damage (Tail % DNA) and (B) average number of  $\gamma$ -H2AX foci in *M. galloprovincialis* and *D. polymorpha* gill cells following exposure to varying concentrations of hydrogen peroxide. SD is standard deviation of mean data. Asterisks (\*, \*\* or \*\*\*) are indicative of significant differences ( $p < 0.05, 0.01, 0.001$ ) from the corresponding control.  $n=4$ .

**Figure 6.** Genotoxic responses in *M. galloprovincialis* and *D. polymorpha* gill cells following a 10 day exposure to copper (Cu). (A) DNA damage (% tail DNA), (B) Induction of micronuclei (MN) and (C) induction of  $\gamma$ -H2AX foci Asterisks (\*, \*\* or \*\*\*) are indicative of significant differences ( $p < 0.05, 0.01, 0.001$ ) from the corresponding control. SD is standard deviation of mean data. Images show (left) control cell and (right) damaged cell.  $n=9$ .

**Figure 7.** Pearson's correlation analyses. Top to bottom: % tail DNA and induction of  $\gamma$ -H2AX foci; % tail DNA and induction of MN and induction of MN and  $\gamma$ -H2AX foci in *M. galloprovincialis* (left) and *D. polymorpha* (right).  $n=9$ .

**Table 1.** Water quality parameters (pH, temperature, salinity and dissolved oxygen [DO], each parameter measured daily [ $n = 10$ ]) and copper concentrations in water ( $\mu\text{g L}^{-1}$ ,  $n = 6$ ). Data are presented as mean  $\pm$  standard deviation (SD).